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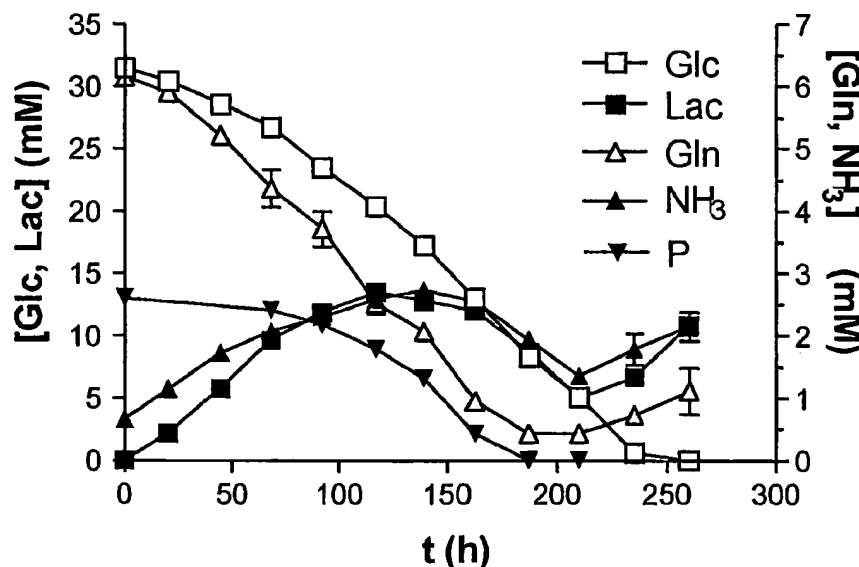
(57) Abstract: The invention provides processes for culturing cells derived from embryonic retinoblast cells immortalized by adenovirus E1 sequences, preferably PER.C6™ cells, to improve product yields from such cells. Feed strategies for such cells and cultures with very high cell densities are provided, resulting in high yields of products, such as recombinant antibodies.

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TITLE OF THE INVENTION

Cultures of E1-immortalized cells and processes for culturing the same to increase product yields therefrom.

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FIELD OF THE INVENTION

The invention relates to the field of cell culture. In particular, the invention relates to the field of culturing cells derived from cells that have been immortalized with E1 sequences from adenovirus. More in particular, the invention

10 relates to culturing such cells to obtain high levels of products from such cells.

BACKGROUND OF THE INVENTION

A human PER.C6® cell line, exemplified by cells deposited at
15 the ECACC under no. 96022940, derived from retina cells by immortalisation with the adenovirus (Ad5) E1a and E1b genes is disclosed in US patent 5,994,128. Besides the ability to function as packaging cells for E1-deleted adenoviral vectors (US patent 5,994,128; WO 01/005945), and for producing other
20 viruses (WO 01/38362), E1-immortalized cells, such as PER.C6 cells, can be used to produce recombinant proteins, such as antibodies (WO 00/63403).
Xie et al (2002) have disclosed a process for serum-free suspension cultivation of E1-immortalized cells. However, the
25 product yields obtained using the culturing processes disclosed in the art for E1-immortalized cells, can be improved. It is an object of the present invention to provide novel processes to increase the product yield from this type of cells.

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BRIEF DESCRIPTION OF THE FIGURES

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Fig. 1. Graph showing growth and antibody production of a
PER.C6 clone (clone 1) grown in shake flask at two different
starting cell concentrations (0.3 and 1.0 x 10^6 ml $^{-1}$). Left
vertical axis: viable cell number (N_v). Right vertical axis:
5   antibody (Ab) concentration. Horizontal axis: time (hours).

Fig. 2. Graph showing the decrease in the cell specific
utilisation of glutamine with increasing viable cell numbers
for an antibody-producing PER.C6 clone (clone 1). N: cell
10   number.

Fig. 3. Profiles for a batch culture of PER.C6 clone 1. A:
Metabolites. Glc, glucose; Lac, lactate; Gln, glutamine; NH_3,
ammonia; P, phosphate. B-E: Amino acids (AA).
15

Fig. 4. Graph showing the effect of a feed component mix
containing glucose, glutamine, amino acids, phosphate, calcium
and growth factors on PER.C6 clone 1. A: viable cell numbers
(N_v). B: antibody (Ab) concentration. Circles: batch. Squares:
20   fed-batch.

Fig. 5. Graph showing viable cell numbers (N_v) and antibody
(Ab) yields of PER.C6 clones where culture medium was
completely exchanged once per day (2 independent experiments
25   each). A: clone 1. B: clone 2.

Fig. 6. Result of modified feed (example 4) for clone 1. A:
viable cell numbers (N_v). B: antibody (Ab) concentration. Open
circles: batch. Closed circles: fed-batch.
30

Fig. 7. Result of further improved modified feed with
different first and subsequent feed additions (example 4,

Table 2) for clone 1. A: viable cell numbers (Nv). B: antibody
(Ab) concentration. Circles: batch. Squares: fed-batch.
Arrows: last feed.

5   Fig. 8. Result of further improved modified feed with
different first and subsequent feed additions (example 4,
Table 2) for clone 2. Nv: viable cell number. Ab: antibody
concentration.

10   Fig. 9. Galactosylation levels of IgG produced according to
processes according to invention.

Fig. 10. Result of further improved modified feed with
different first and subsequent feed additions (example 4,
15   Table 2) for clone 3. Nv: viable cell numbers. Ab: antibody
concentration.

SUMMARY OF THE INVENTION

20   In one aspect the invention provides feed strategies for fed-
batch or fed-perfusion cultures of cells immortalized by
adenovirus E1 sequences. In one embodiment thereof the
invention provides a method for the culturing of such cells,
said cells capable of growing in suspension, comprising the
25   steps of: determining at least once during the culturing of
the cells the concentration of at least one medium component
selected from the group consisting of glucose, glutamine
phosphate, leucine, serine, isoleucine, arginine, methionine,
cystine, valine, lysine, threonine and glycine, adding
30   components to the medium during the culturing of the cells at
or prior to the depletion of at least one of the components of
which the concentration was determined in the previous step,

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wherein the components added at least comprise glucose,
glutamine, phosphate, leucine, serine, isoleucine, arginine,
methionine and cystine. Other components that beneficially may
be added according to the invention, amounts and time of
5   addition of the components are provided herein below, as well
as in the claims.
It is another aspect of the invention to provide a culture of
cells derived from cells immortalized by adenovirus E1
sequences, characterized in that said culture comprises at
10   least 10 x 10^6 cells/ml. Preferably, said culture comprises at
least 12 x 10^6 cells/ml, more preferably at least 15 x 10^6
cells/ml. In certain preferred embodiments the culture
according to the invention comprises more than 20 x 10^6, 25 x
10^6, 30 x 10^6 or 40 x 10^6 cells/ml. Methods to obtain such
15   cultures are also provided herein.
In yet another aspect, a method to increase cell densities and
product yields from a culture of cells immortalized by
adenovirus E1 sequences is provided. In one embodiment hereof,
a process for culturing such cells is provided, characterized
20   in that said process comprises a step of subculturing said
cells at a seeding concentration of between 0.8 x 10^6 and 2.0 x
10^6 viable cells/ml, preferably between 0.9 x 10^6 and 1.5 x 10^6
viable cells/ml.
Preferably, the cells used in the methods of the invention are
25   derived from retina cells, more preferably from human
embryonic retina (HER) cells, such the cells deposited under
ECACC no. 96022940. In certain embodiments, said cells are
PER.C6 cells.
In certain embodiments, said cells can produce recombinant
30   proteins, preferably antibodies, at high yields. In other
embodiments said cells comprise recombinant adenoviral vectors
having a deletion in the E1-region, or other viruses, which

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can be produced on said cells in high yields using the process
according to the invention. In preferred embodiments, the
cells are cultured at least part of the time in a serum-free
medium.

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DETAILED DESCRIPTION OF THE INVENTION

The productivity of any cell line is mainly defined by three
basic parameters, the specific productivity of the cell line,
the peak viable cell concentration that is attainable and the
10   length of the production process that is possible. Increases
in either of these variables will lead to increases in the
final product concentration and is dependent to a large extent
on the cell line. In a straight batch culture, cell lines such
as CHO and SP2/0 can achieve cell densities up to 4×10^6/ml.
15   In fed-batch or perfusion processes the viable cell
concentration is increased, and typically hybridoma cells such
as SP2/0 can be cultured up to 10×10^6 cells/ml, while CHO can
be cultured up to $6-10 \times 10^6$ cells/ml. The invention describes
methods to increase the viable cell density of cultures of
20   cells immortalized by adenovirus E1 sequences, preferably
derived from embryonic retina cells, to attain cell densities
beyond those reported in the prior art. Furthermore, the
methods according to the invention can be used to obtain
higher product yields from cultures of cells according to the
25   invention.

The present invention discloses improvements in how E1-
immortalized cells, such as PER.C6 cells, can advantageously
be used for the production of high yields of monoclonal
30   antibodies. It is disclosed that these cells be cultured to
very high viable cell concentrations in a straight batch
process (up to 14×10^6 viable cells/ml).

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Furthermore, E1-immortalized cells, such as PER.C6 cells, are well suited to a fed-batch process as a culture of these cells unexpectedly consumes lactate and ammonia and maintains viability for long periods of time under nutrient limiting

5   conditions. Methods to increase product yields from said cells by a feed strategy in cultures are provided herein.

With the term 'feed strategy' as used herein is meant the addition of certain identified components including but not limited to nutrients, such as sugars, amino acids, and the

10   like, to the culture medium. The identified components are preferably added in certain amounts and at certain times, when they are required to improve product yields from the cells, such as provided herein.

E1-immortalized cells, such as PER.C6 cells, are also

15   well suited to a perfusion process as they can be maintained at very high viable cell concentrations (up to 50×10^6 cells/ml with a viability of at least 85%) for long periods of time and with good final product concentrations.

20   Culture media

The processes of the invention generally increase the product yields from the cells compared to yields obtained with processes described in the art for the cells according to the invention. Preferably, serum-free culture media are used at

25   least part of the time in the processes according to the invention. Preferably, the medium contains only recombinantly produced proteins, which are not of animal origin. Such culture media are commercially available from various sources. In one embodiment of the invention, VPRO culture medium (JRH

30   Biosciences) is used for the fed-batch or (fed-)perfusion process.

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Products

The methods of the invention are preferably used to
produce products in cells of the invention. The processes of
the present invention can be used for the improved production

5   of antibodies, as well as other proteins (WO 00/63403). For
the production of proteins, the cells of the invention
suitably comprise nucleic acid encoding said proteins, in
operable association with elements capable of driving
expression of said proteins. Furthermore, the processes can be

10   used for improvement of the production of recombinant
adenoviral vectors having a deletion in the E1-region, in
which case the cells are used as complementing cells, which in
itself is known to the skilled person according to established
methodology (e.g. US patent 5,994,128; WO 01/005945).

15   Moreover, the processes according to the invention can be used
to improve a process for propagation of other (non-adenovirus)
viruses in the cells (WO 01/38362). Hence, products according
to the invention can be recombinant proteins, such as
antibodies, erythropoietin, and the like, as well as

20   recombinant adenoviral vectors with a deletion in the E1
region, or other viruses.

Cells

The cells according to the invention are cells that have

25   been immortalized with E1 sequences from an adenovirus, which
cells are also referred to herein as E1-immortalized cells.
Such cells express at least a functional part of the E1A
region of an adenovirus, and preferably also at least a
functional part of the E1B region. E1A protein has

30   transforming activity, while E1B protein has anti-apoptotic
activities. The cells according to the invention may be
derived from any cell, including lung cells, kidney cells,

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amniocytes, but preferably are derived from retina cells. They
may be derived from embryonic retina cells. Preferably the
cells according to the invention are human cells. A method for
immortalization of embryonic retina cells has been described

5   in the art (US patent 5,994,128). Accordingly, a retina cell
that has been immortalized with E1 sequences from adenovirus
can be obtained by that method. In certain preferred
embodiments, the cells of the invention are derived from E1-
immortalized HER cells, such as PER.C6 cells. PER.C6 cells for

10   the purpose of the present application shall mean cells from
an upstream or downstream passage or a descendent of an
upstream or downstream passage of cells as deposited under
ECACC no. 96022940. In addition, also the E2A region with a
ts125 mutation may be present (see e.g. US patent 6,395,519)

15   in said cell. A cell derived from a PER.C6 cell can be a
PER.C6 cell infected with recombinant adenovirus or other
virus, and can also be a PER.C6 cell into which recombinant
nucleic acid has been introduced, e.g. comprising an
expression cassette wherein nucleic acid encoding a protein of

20   interest is operably linked to sequences capable of driving
expression thereof, such as a promoter and polyA signal,
wherein preferably said cells are from a stable clone that can
be selected according to standard procedures known to the
person skilled in the art. A culture of such a clone is

25   capable of producing a protein encoded by said recombinant
nucleic acid.

        Components for feed strategies
        In one aspect, the invention provides processes for

30   culturing cells according to the invention, wherein by feed
strategies according to the invention certain amino acids are
added during the culturing process to replenish amino acids of

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which the concentration has become or will become limiting for
an optimal process and product yields. By amino acid is
intended all naturally occurring alpha amino acids in both
their D and L stereoisomeric forms, and their derivatives. A
5   derivative is defined as an amino acid that has another
molecule or atom attached to it. Derivatives would include,
for example, acetylation of an amino group, amination of a
carboxyl group, or oxidation of the sulfur residues of two
cysteines to form cystine. Further, amino acid derivatives may
10   include esters, salts, such as chlorides, sulphates, and the
like, as well as hydrates. It will be understood by the person
skilled in the art, that where a specific amino acid is
mentioned herein, a derivative may also be used and is meant
to be included within the scope of the invention. Other
15   components such as sugars, growth factors, vitamins, etc may
also be added to improve the processes according to the
invention.
     Feed strategies
     In one aspect, the invention provides a method for
20   producing a product in cells immortalized by adenovirus E1
sequences, in a culture medium, wherein said product is chosen
from the group consisting of a recombinant protein, a virus,
and a recombinant adenovirus with a deletion in the E1 region,
characterized in that said method comprises a step wherein at
25   least Leucine, Serine, Isoleucine, Arginine, Methionine and
Cystine are added to the culture medium. In one aspect the
invention provides a method for the culturing of cells
immortalized by adenovirus E1 sequences, said cells capable of
growing in suspension, comprising the steps of: determining at
30   least once during the culturing of the cells the concentration
of at least one medium component selected from the group
consisting of glucose, glutamine, phosphate, leucine, serine,

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isoleucine, arginine, methionine, cystine, valine, lysine,
threonine and glycine, adding components to the medium during
the culturing of the cells at or prior to the depletion of at
least one of the components of which the concentration was
5   determined in the previous step, wherein the components added
at least comprise glucose, glutamine, phosphate, leucine,
serine, isoleucine, arginine, methionine and cystine.
"Depletion" as used herein is defined as the time a component
has a concentration of 30% or less of the starting
10   concentration in the culture medium. In these aspects, the
determination of the concentration of at least one medium
component selected from the group consisting of glucose,
glutamine, phosphate, leucine, serine, isoleucine, arginine,
methionine or cystine is preferred over the determination of
15   only components selected from the group consisting of valine,
lysine, threonine and glycine. In certain embodiments, the
concentration of at least two medium components according to
the invention is determined in the first step. In certain
embodiments, the components that are added further comprise
20   one or more of valine, lysine, threonine, glycine, asparagine,
tyrosine, histidine, phenylalanine, tryptophane, calcium,
LongR3 IGF-1, Long EGF and insulin. In specific embodiments,
the components are added in an end concentration in mmoles/l
of freshly added component per 10×10^6 cells/ml of 6.0 for
25   glucose, 2.60 in the first feed and 1.75 in subsequent feeds
for glutamine, 0.70 for phosphate, 0.66 for leucine, 1.10 in
the first feed and 0.55 in subsequent feeds for serine, 0.50
for isoleucine, 0.46 for arginine, 0.23 for methionine, and
0.25 for cystine. In further embodiments, the following
30   components are further added to an end concentration in
mmoles/l of freshly added component per 10×10^6 cells/ml of
0.45 for valine, 0.44 for lysine, and 0.30 for threonine. In

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further embodiments, the following components are further
added to an end concentration in mmoles/l of freshly added
component per 10 x 10^6 cells/ml of 0.10 for asparagine, 0.13
for tyrosine, 0.10 for histidine, 0.02 for phenylalanine, and
5   0.06 for tryptophan. Furthermore, calcium may be added in an
end concentration in mmoles/l of freshly added component per
10 x 10^6 cells/ml of 0.02. Growth factors such as IGF, EGF, and
insulin or their derivatives may also suitable be present in
the growth medium. The amounts for the addition of components
10   above may have an error margin per component of 33% or less,
preferably 20% or less, more preferably 10% or less, even more
preferably 5% or less. The amounts are presented per 10 x 10^6
cells/ml, and are linearly dependent on the number of
cells/ml. In preferred embodiments, said components are added
15   at between 48 hours and the moment of depletion of at least
one of the medium components the concentration of which was
determined in the previous step. In certain embodiments, said
addition is at a time between 24 hours and just prior to
depletion. In certain aspects, the invention provides a method
20   according to the invention, wherein said cells express a
recombinant immunoglobulin that is secreted into the culture
medium to a level of at least 500 mg per liter, preferably at
least 700 mg/l, more preferably at least 850 mg/l, even more
preferably at least 1000 mg/l, still more preferably at least
25   1250 mg/l, still more preferably at least 1500 mg/l, still
more preferably at least 1750 mg/l and still more preferably
at least 2000 mg/l. In general, the addition of medium
components according to the invention, i.e. in for instance a
fed-batch process, results in an increase in the yield of
30   produced product of at least 1.5x, preferably at least 2x,
more preferably at least 2.5x and still more preferably about

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3x or even higher, compared to the process wherein no
components are added, i.e. the batch process.

In addition to use in a fed-batch process, the feed
strategies of the invention can also be beneficially used in
5   an optimized batch process, as set out in example 5.

    Perfusion
    Alternatively, in another aspect of the invention the
entire culture medium may be exchanged. It is shown that
10 unexpected high viable cell densities can be attained when
this is applied to cells derived from retina cells
immortalized by adenovirus E1 sequences. Exchanging culture
medium may be performed by any means known to the person
skilled in the art, including but not limited to collection of
15 the cells by centrifugation, filtration, and the like,
followed by re-suspension of the cells into fresh culture
medium. Alternatively, a perfusion system may be used, wherein
culture medium is either continuously or intermittently
exchanged using a cell separation device such as a centritech
20 centrifuge or passage through a hollow fibre cartridge, and
the like. It is therefore another aspect of the invention to
provide a process for culturing cells derived from embryonic
retina cells immortalized by adenovirus E1 sequences,
characterized in that culture medium is exchanged at a rate of
25 0.2-3, preferably 0.5-3, culture volumes per day (24 hours).
Cultures obtained using this method preferably have viable
cell densities higher than 20 x 10^6 cells/ml, more preferably
higher than 30 x 10^6 cells/ml. In certain aspects, such
cultures have cell densities higher than 40 x 10^6 cells/ml. In
30 certain aspects such cultures are used to produce recombinant
antibodies with a yield of at least 150 mg/l/day, preferably
at least 200 mg/l/day, more preferably at least 300, 400, or

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500 mg/l/day. Of course, also other products according to the invention can be produced by such methods. It is shown in here that one complete volume exchange of culture medium each day supports at least 30×10^6 viable cells/ml with antibody yields

5   of more than 500 mg/L/day (up to 750 mg/l/day) (Fig. 5). One complete medium exchange per day corresponds to a continuous perfusion rate of 3 volumes per day, meaning that a continuous perfusion system could yield approximately at least 150-200 mg/L/day. One method to reduce this perfusion rate and thus

10 increase antibody yields (by reducing the volume in which the antibody is secreted) is to supplement the fresh culture medium with the essential components (known as fed-perfusion). These components for antibody-producing E1-immortalized cell, such as PER.C6 cell, clones are identified herein (see example

15 2) and therefore it is another aspect of the present invention to provide such a fed-perfusion system, wherein the feed strategies according to the present invention are employed. A common drawback of fed-perfusion processes is the build-up of toxic metabolic by-products (such as lactate and ammonia),

20 which can result in low cell viabilities and product yields. There is often a requirement at high cell concentrations for a high perfusion rate to remove these by-products. One advantage demonstrated for E1-immortalized cell, such as PER.C6 cell, clones according to the invention is that they are capable of

25 utilising lactate and ammonia such that concentrations do not become problematical (see Fig. 3A). It is therefore possible to obtain an antibody yield of at least 500 mg/l/day by changing the culture medium once or twice a day. Alternatively this can be achieved by using a continuous perfusion rate of

30 for instance 1 volume per day in combination with supplementation of the medium with a feed concentrate (fed-

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perfusion). This can advantageously be combined with a cell
bleed (removing a certain percentage of the cells population).

Cultures with high cell densities are advantageous for
obtaining high product yields. It is therefore another aspect

5   of the invention to provide a culture of cells derived from
cells immortalized by adenovirus E1 sequences, said culture
comprising at least 10×10^6 cells/ml. The viability in the
culture is at least 80%. Preferably, the viability is at least
90%, more preferably at least 95%. The cultures according to

10 the invention are preferably suspension cultures, meaning that
the cells in said cultures are in suspension in the culture
medium, such as in shake flasks, roller bottles, bioreactors,
including stirred tanks, air lift reactors, and the like. The
strategies disclosed herein may however also be used for

15 cultures of cells in hollow fiber reactors, such as described
by Tanase et al (1997), and for adherent cultures, such as
cells on microcarriers. In one embodiment, said culture
comprises at least 12×10^6 cells/ml. It is disclosed herein
that up to 14×10^6 cells/ml can be obtained by a straight

20 batch culture.

It is further demonstrated that, using medium perfusion,
even higher cell densities can be achieved, up to 50×10^6
cells/ml. The prior art does not provide any indication that
such unexpected high cell densities are obtainable. In other

25 preferred embodiments therefore, the invention provides a
culture of cells derived from E1-immortalized cells,
preferably derived from retina cells, said culture comprising
at least 15×10^6 cells/ml, preferably at least 20×10^6
cells/ml, more preferably at least 25×10^6 cells/ml. In

30 specific embodiments, said culture comprises at least 30×10^6
cells/ml, or even at least 40×10^6 cells/ml. Cultures with at
least 15×10^6 cells/ml according to the invention appear

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obtainable by a perfusion process, meaning that culture medium
is exchanged during the culturing process. The cultures
according to the invention have a viability of at least 80%,
preferably at least 85%, more preferably at least 90%, still
5   more preferably at least 95%. Said cultures are suspension
cultures. Said cultures further comprise growth medium. Said
growth medium preferably is serum-free. The cells of the
culture may comprise recombinant nucleic acid molecules
encoding immunoglobulins, or parts or derivatives thereof, in
10 expressible format. Such cells are capable of producing
immunoglobulins in high yields. In particular, it is shown
herein that a culture of cells according to the invention,
wherein the medium is exchanged every day, and wherein more
than 30×10^6 cells/ml are present, can provide recombinant
15 antibody yields of at least 500 mg/l/day. The cells in said
culture preferably produce at least 10 pg protein/cell/day.

The processes of the invention, especially those for
recombinant protein production, can also be combined with
20 other measures described in the art that in some cases improve
product yields. Therefore, in certain embodiments of the
invention the culture medium is subjected to a temperature
shift before or during the production phase, e.g. by running
the process at a lower temperature, e.g. between 30°C and
25 35°C, in the production phase (see e.g. US patent 6,506,598,
and literature cited therein, which describes effects of
lowering the cell culture temperature on several parameters
for recombinant protein production), or by the addition of
cold culture medium to the culture (wherein cold is meant to
30 be lower than the temperature the cells are cultured in,
preferably the cold culture medium having a temperature
between 2°C and 8°C) when the cells are subcultured or later

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during the culture process. In other embodiments, specific
growth factors may be added to improve the processes according
to the invention with regard to product yields. In yet other
embodiments for the production of proteins, the processes
5   according to the invention may be improved by the addition of
alkanoic acids or salts thereof, such as sodium butyrate,
either during the whole culture phase or only during the
production phase (see e.g. US 6,413,746, and references
therein, which describes effects of addition of butyrate on
10   production of proteins in cell culture). In yet other
embodiments for the production of proteins, the culture medium
is subjected to a temperature or pH shift (Weidemann et al
1994, Sauer et al 2000).

15   It will be clear to the person skilled in the art that
several aspects and/or embodiments according to the invention
can be combined to provide a process for culturing cells which
leads to particularly good product yields. As a non-limiting
example, it is for instance possible to seed a culture of E1-
20   immortalized cells at about 0.8×10^6 to 2.0×10^6 cells/ml,
and use a feed strategy and/or exchange the growth medium
during the culturing process to improve the final product
yields.
The invention will now be illustrated with some examples, not
25   intended to limit the scope of the invention.

Experimental
Methods and vectors for genetically engineering cells and/or
cell lines to express a protein of interest are well known to
30· those of skill in the art; for example, various techniques are
illustrated in Current Protocols in Molecular Biology, Ausubel
et al., eds. (Wiley & Sons, New York, 1988, and quarterly

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updates) and Sambrook et al., Molecular Cloning: A Laboratory
Manual (Cold Spring Laboratory Press, 1989). General and
standard cell culture techniques are known to the person
skilled in the art, and are for instance described in R.I.

5   Freshney, Culture of animal cells: A manual of basic
technique, fourth edition (Wiley-Liss Inc., 2000, ISBN 0-471-
34889-9). Such standard techniques were followed unless
otherwise noticed.

10 Cell Culture Protocols
PER.C6 cells were cultured in the examples. Cells were adapted
from adherent cultures in DMEM containing 10% FBS (Invitrogen)
to serum free medium by direct transfer. Briefly, sub-
confluent, logarithmic cells were trypsinised, washed once

15 with serum free medium and inoculated directly into 250 ml
Ehrlenmeyer flasks with a 0.2μ filter (Corning), containing 25
ml of ExCell-525 serum-free medium (JRH Biosciences) at a
starting cell concentration of $0.3-0.5 \times 10^6 \text{ ml}^{-1}$, unless
otherwise noted. Cultures were maintained in logarithmic

20 growth in Ehrlenmeyer flasks by passage every 2-3 days. Flasks
were shaken on a magnetic shaker platform (Infors) at 100 rpm
in a humidified incubator at 37°C and 5% CO_2. Cultures were
passaged by centrifugation at 1000 rpm for 5 minutes. The
supernatant was removed and the pellet re-suspended in the

25 remaining medium. Fresh, cold medium (4°C) was added and new
flasks inoculated at the appropriate cell concentration. After
transfer to serum-free medium, cultures were passaged for 2-4
weeks to allow for complete adaptation, after which a serum-
free cell bank was created. All experiments were started using

30 cells from this cell bank.

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Bioreactors

Bioreactor cultures were performed in 3L reactors with a 2L
working volume (Applikon). Temperature was maintained at 37°C
by a heating blanket. Dissolved oxygen concentration (dO_2) was
5   controlled at 50% of air saturation by adjusting inlet gas
composition through the headspace and intermittent sparging
through a microporous sparger. Starting culture pH was
controlled at 7.3 by CO_2 addition through the microporous
sparger. The lower culture pH limit was set at 6.7 so that the
10   culture pH was allowed to drift downwards (the lower limit was
not reached). Cultures were agitated by two marine impellers
at 75 rpm. Process data was acquired by the BioExpert software
(Applikon).

15   Analytical Protocols

Cell counts and viability measurements were performed using a
CASY automatic cell counter (Schärfe Systems). Glucose,
lactate, ammonia and phosphate concentrations were determined
using an Ektachem II analyser (Kodak) with cell-free culture
20   supernatants. Amino acid concentrations were determined using
a modified AccuTag HPLC method (Waters) as described by van
Wandelen and Cohen (1997). Aliquots (200 µl) of centrifuged
culture supernatant were stored at -20°C in 1 ml cryovials
(Nalgene) until required. Samples from each experiment were
25   analyzed at the same time to avoid experimental variation.
Osmolality was measured by a freezing point depression
osmometer (Osmomat 030-d, Gonotec). Antibody concentration was
determined by a sandwich-type ELISA. Biefly, plates were
coated with 2 µg ml^{-1} mouse anti-human IgG against the kappa
30   light chain (Pharmingen) and incubated overnight at 4 °C. An
HRP-conjugated mouse anti-human IgG against the heavy chain
(Pharmingen; 1:500) was used as detection antibody for 1 hr at

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37 °C with OPD (Sigma) as substrate. Washing between
incubation steps was performed with 0.05 % Tween 20 in PBS.
Samples were diluted in washing buffer supplemented with 0.1 %
BSA. Quantification was relative to an IgG1 reference standard
5   using a calibration range of 10 to 400 ng ml^{-1}. Antibody
samples purified by Protein A were subject to quality analysis
by isoelectric focusing (IEF) and denaturing polyacrylamide
gel electrophoresis (SDS-PAGE). For glycan analysis, N-linked
glycans were removed by PNGase F treatment of the IgG samples
10   in 20 mM sodium phosphate (pH 7.2) and analyzed with MALDI-MS
in the reflector mode on an Applied Biosystems Voyager DE Pro
mass spectrometer. The matrix was 2,5-dihydroxybenzoic acid
(10 mg ml^{-1}) in 50/50/0.1 acetonitrile/water/trifluoroacetic
acid. Spectra were obtained in the positive ion mode and
15   glycans were detected as sodium adducts, [M+Na]+.

Calculation of Cell Specific Metabolic Rates
Cell specific rates of metabolite utilisation and production
in batch and fed-batch culture were calculated using the log
20   mean of the cell concentration as shown in the following
equation:
$q_s = (C_2 - C_1) / (t_2 - t_1) \times [(X_2 - X_1) / \ln(X_2 - X_1)]$.
In this equation, C is the metabolite concentration
(μmoles/l), t is time (days) and X is the viable cell
25   concentration. A rate constant accounting for the spontaneous
decomposition of glutamine was not included as decomposition
was not significant at the time points at which the rates were
calculated (data not shown). The yield coefficients of lactate
produced per glucose ($Y_{lac/glc}$), ammonia produced per glutamine
30   ($Y_{amm/gln}$) and alanine produced per glutamine ($Y_{ala/gln}$) were
calculated from the equations below and are expressed in
mole/mole:

20

$$Y_{lac/glc} = q_{lac} / q_{glc}$$

$$Y_{amm/gln} = q_{amm} / q_{gln}$$

$$Y_{ala/gln} = q_{ala} / q_{gln}$$

5

EXAMPLES

Example 1: Increasing maximum final cell yields in batch
culture of PER.C6 cells

10 The simplest production process is a batch culture.
However, this is restricted in the viable cell concentration
and therefore the product yields attainable, due largely to
nutrient limitation. A method is presented to increase the
maximum final cell concentration of a batch culture of PER.C6
or PER.C6 derived sub-clones by calculating the cell specific
15 rate of utilisation of key nutrients at different cell
concentrations and starting the batch culture at a cell
concentration where there is optimal utilization of nutrients
with respect to cell growth.

The DNA encoding the antigen-binding region of an
20 antibody recognizing epithelial cell adhesion molecule (EpCAM)
was first isolated from a scFv phage display library (Huls et
al, 1999). DNA encoding the antigen-binding region of an
antibody recognizing CD46 was isolated as disclosed in WO
02/018948. A leader sequence and constant regions of IgG1 type
25 were added essentially as described in Boel et al, 2000. The
DNA encoding the light and heavy chains were then cloned into
expression vector pcDNA3002(Neo). The expression vector
pcDNA3002(Neo), which has been described in international
patent application PCT/NL02/00841, was deposited on December
30 13, 2001 at the European Collection of Cell Cultures (ECACC)
under number 01121318. The resulting expression vectors,
encoding an IgG1 that recognizes EpCAM or CD46, respectively,

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regulated by a CMV promoter, was introduced in PER.C6 cells
according to standard methods.
A recombinant antibody-expressing clone, derived from a
parental population of the PER.C6 cell line, was used in these
5   experiments. The clone expressing anti-EpCAM is further
referred to herein as clone 1, the clone expressing anti-CD46
is further referred to herein as clone 2.
Cells were maintained in ExCell™ 525 medium (JRH Biosciences)
(maintenance of the cells in GTM-3 medium (Sigma) did also
10   work) and batch productions were carried out in ExCell™ VPRO
medium (JRH Biosciences, Cat. No. 14560). Cells were
transferred directly from ExCell™ 525 to ExCell™ VPRO for the
batch productions.
Fig. 1 shows that the maximum final viable cell
15   concentration of cultures started at 1×10^6 cells ml^{-1} reached
almost 14×10^6 cells ml^{-1} after 6 days (approximately 3-fold
higher than batch cultures of CHO and Sp2/0), compared to
cultures started at 0.3×10^6 ml^{-1}, which reached 10×10^6 cells
ml^{-1} after 9 days. There is very little difference in the final
20   antibody titres of both cultures. However, in the culture
started at 1×10^6 ml^{-1}, approximately 600 mg L^{-1} was reached
after 6 days, compared to 9 days for the cultures started at
0.3×10^6 ml^{-1}.
The higher cell concentrations observed in cultures
25   started at 1×10^6 cells ml^{-1} compared to 0.3×10^6 ml^{-1} is due
to the lower cell specific rate of nutrient utilisation at the
higher cell concentration. The respiration rate of hybridoma
cells has been shown to decrease with increasing cell density
(Wohlpart et al 1990). Similarly, the cell specific rate of
30   utilisation of a nutrient has also been shown to decrease with
increasing cell concentration (Portner et al 1994, Yallop and
Svendsen 2001). We have now used this information in a novel

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and inventive way to form a concept for increasing attainable
cell densities in a culture.

By calculating the cell specific rate of utilisation of a
key nutrient each day in a batch culture and plotting these
5   values against cell concentration, a graph can be obtained as
shown in Fig. 2 for glutamine. Fig. 2 shows the relationship
between the cell specific rate of glutamine utilisation (q_{Gln})
and cell concentration. From this graph, an optimum starting
cell concentration can be selected based on optimal use of the
10   available nutrients. For example, a culture starting at 0.3 x
10^6 cells ml^{-1} will reach approximately 0.5 x 10^6 ml^{-1} in 24h
(average population doubling time (pdt) of this clone is 32h).
The q_{Gln} value at 0.5 x 10^6 cells ml^{-1} is approximately 2.5
μmoles 10^6 cells^{-1} 24h^{-1}. The total glutamine consumed in this
15   24h will therefore be approximately 1.25 μmoles ml^{-1} (0.5 x
2.5). However, a culture starting at 1 x 10^6 cells ml^{-1} will
reach approximately 1.5 x 10^6 ml^{-1} in 24h. The q_{Gln} value at
this cell concentration is approximately 0.75 μmoles 10^6 cells^{-1}
24h^{-1}. The total glutamine consumed will therefore be
20   approximately 1.125 μmoles ml^{-1}. The two cultures will
therefore use approximately the same amount of glutamine in
the first 24h.

It is therefore another object of the invention to
provide a method of culturing cells, comprising starting a
25   culture at a cell concentration where the specific nutrient
utilization level is close to a minimum plateau level. This
equates with around 0.8 to 2.0 x 10^6 cells/ml, preferably 0.9 –
1.5 x 10^6 cells/ml, for E1-immortalized retina cells,
particularly PER.C6-derived cells. It is therefore an
30   embodiment of the invention to subculture the cells at a
seeding concentration of 0.8-2.0 x 10^6 cells/ml, preferably

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0.9-1.5 x 10^6 cells/ml, more preferably 0.95-1.25 x 10^6 cells/ml.

The advantage of this aspect of the invention is that the number of viable cells that can be obtained is higher at this
5 higher seeding density, and higher numbers of cells are reached faster during the process. This aspect of the invention therefore is very useful for batch cultures, but can also be beneficially used in fed-batch cultures or (fed-) perfusion cultures, such as those of the present invention.
10
Example 2: Feed Strategies for improving antibody yields in PER.C6 derived sub-clones.

Fed-batch processes aim at increasing product yields by increasing the viable cell concentration or prolonging the
15 production period by feeding nutrient concentrates to replenish those that are consumed. We present here a feed strategy for improving the antibody yields of PER.C6 derived sub-clones. The feed strategy can be combined with a higher starting cell density to obtain a higher final cell density at
20 the onset of the nutrient feed and a shorter overall production process.

A basic nutrient feed concentrate consisting of glucose, phosphate, glutamine and the 15 other amino acids was prepared based on the nutrient utilisation profile of six duplicate
25 batch cultures of clone 1 in shake-flask (see e.g. Fig. 3). Similar utilization profiles were observed for clone 2, and hence it is expected that the feed strategy described below for clone 1 will also improve yields from other clones, thereby providing a more generic strategy for fed-batch or
30 fed-perfusion cultures of E1-immortalized cells, preferably retina cells, preferably cells derived from PER.C6 cells. The concentrate is listed in Table 1. Optionally, calcium and

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three recombinant growth factors, LongR3 IGF-1, Long EGF and
insulin were also added to the feed. At this point, the
addition of calcium and the growth factors did not
significantly influence the results that were obtained.

5   Glycine appeared not essential for the feed, and was no longer
added in later experiments. Insulin was purchased from Sigma,
LongR3 IGF-1 and Long EGF were purchased from GroPep. All
amino acids were purchased from Sigma. The timing and
frequency of addition of the feed concentrates was varied. The

10   time of the first addition was tested at 0, 1 and 2 days prior
to nutrient exhaustion. Glucose and phosphate were used as
indicators for the start of the feed. A series of bolus
additions were made every two days, based on the predicted
viable cell concentration. Usually, 6 feeds were provided. The

15   concentrations of the added components as presented in Table 1
do not take into account the remaining component in the spent
medium before the addition (i.e. the concentration of a
component after addition into the culture medium will be
higher than that provided in the Table, because before the

20   addition the culture medium will still contain some of this
component, as additions according to the invention are done
before the component is completely used up by the cells).

     Fig. 4 shows the effect of feeding the concentrate mix to
a sub-clone of PER.C6 expressing a recombinant antibody (clone

25   1). Starting the feed at day 3 (two days prior to nutrient
exhaustion and continuing every two days after this) resulted
in a final antibody yield of approximately 800 mg L^{-1}, an
increase of approximately 1.6-fold over the batch process,
which gave 500 mg L^{-1}. Starting the feed at day 5 and

30   continuing every two days after this) resulted in a similar
increase in final antibody concentration.

25

Osmolality in the batch cultures (example 1) decreased from 280 to 240 mOsm Kg^{-1}, while in the feed cultures, it increased, eventually rising to 300-310 mOsm Kg^{-1}.

5   **Example 3**: Achieving viable cell numbers above 30 x 10^6 cells per ml and antibody yields above 500mg L^{-1} day^{-1}

Fed-batch processes may result in a build-up of toxic metabolites such as lactate and ammonia and an increase in medium osmolarity, which eventually limit the viable cell
10 concentration and the length of the process, thus impacting on product yields. A possible alternative to a fed-batch process is a perfusion process, where high cell concentrations can be maintained by a continual medium exchange and a cell bleed (removing a certain percentage of the cells population). A
15 possible drawback with such a process is a relatively low product concentration due to the large volumes of medium that are required, the relatively low cell viability often encountered and the relatively high level of complexity to operate such a system. It is therefore only advantageous to
20 operate perfusion processes if very high viable cell concentrations and/or specific productivities can be maintained.

We present here the attainment of a viable cell concentration above 30 x 10^6 ml^{-1} and antibody yields of above 600 mg L^{-1} $24h^{-1}$
25 in shake flask cultures with one medium volume exchange per day.

Logarithmic cultures of antibody producing PER.C6 cells, cultured in shake flask with $ExCell^{TM}$ 525 were transferred into shake flasks containing $ExCell^{TM}$ VPRO at a starting cell number
30 1 x 10^6 cells/ml (other starting cell concentrations gave similar results). Medium replacement by centrifugation (one volume per day) was started at day 3-5. No cell bleed was

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operated. Samples for metabolite analysis, antibody
quantification and cell counts were taken every day and stored
at −20°C.

Fig. 5 shows that a viable cell number of up to 50×10^6
5   ml^{-1} and an antibody yield of 500-750 mg L^{-1} $24h^{-1}$ was
maintained for at least 5 days without a cell bleed, for two
independent antibody producing cell clones. Viability of the
cells was around 80-90%. These high cell densities are
approximately 3-fold higher than is generally achievable with
10   other cell lines like CHO and Sp2/0, and hence retina cells
that are immortalized with adenovirus E1 sequences, such as
PER.C6 cells, are very suitable for perfusion processes. A
cell bleed will improve the length of the process, and
therefore an optimized system may include one or more cell
15   bleed steps.

Up to 50×10^6 cells per ml, with a viability of around
80-90%, could be maintained for at least 5 days with one
complete medium change every two days. With this strategy,
many of the nutrients became depleted on the second day. The
20   medium is therefore preferably changed daily. In a perfusion
process, this could translate into a change of about 1-3
volumes/day. This is near the typical range in a standard
perfusion system, where the medium is changed at about 0.5 to
2 volumes/day. The somewhat higher values for the cells
25   according to the invention are due to the very high cell
concentrations with the cells of the invention in a perfusion
system. When cell concentrations of more than 30×10^6 cells/ml
according to the invention are preferred, the medium exchange
should at least be 0.5 culture volumes/day, preferably at
30   least 1 culture volume/day. Failure to supply the nutrients
(here via the culture medium) in sufficient concentration
leads to cell death. The daily medium change results in higher

viable cell densities (up to 50 x 10^6 cells/ml with daily
medium change vs. 10 x 10^6 cells/ml without daily medium
change, see Figs 1 and 4). Furthermore, with a daily medium
exchange, the cells give similar product yields in one day as
5   achieved in a batch process of 8-13 days.

Example 4: Feed Strategies for further improving antibody
yields in PER.C6 derived sub-clones.
The provision of a balanced nutrient feed extends to
10 components such as vitamins, trace elements and lipids.
Concentrates (10x or 50x, both worked) of ExCell VPRO
vitamins, inorganic salts, trace elements, growth factors,
lipids and plant hydrolysates were obtained from JRH
Biosciences and added together with the basic feed concentrate
15 (minus calcium and growth factors) described in example 2. The
ExCell VPRO concentrates were added to give a final
concentration of 0.25X.
Fig. 6 shows the results of this modified feed on the growth
(Fig. 6A) and antibody yields (Fig. 6B) of clone 1 in shake-
20 flask versus a batch control. The results were obtained by
starting the feed at day 3 (48h prior to nutrient depletion).
Starting the feed at day 5 (day of nutrient depletion) gave
similar results. The viable cell number was maintained for
significantly longer than the batch control and antibody
25 yields increased 2.0-fold from 0.5 g L^{-1} in the batch to 1.0 g
L^{-1} in the fed-batch process.

Spent medium analysis of these feed experiments identified a
change in the cell specific rates of utilization of some of
30 the amino acids, which appeared to be de to the addition of
the VPRO concentrates. The amino acid concentrate listed in
Example 2 was therefore modified as shown in Table 2. The feed

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was started 48h prior to nutrient depletion and additions were
made every two days. Usually, 6 feeds were provided. Again,
the concentrations of the added components as presented in the
Table do not take into account the remaining component in the
5   spent medium before the addition.
For the first feed addition, increased concentrations of
Glutamine and Serine were used as compared to the subsequent
feeds (see Table 2). Phosphate and glucose were used as
markers to determine the start of the feed. Clones 1 and 2
10   were used in this experiment.
Experiments were carried out in shake-flask and bioreactor.
Shake flask experiments were carried out as described.
Bioreactor experiments were initiated by inoculating a 3L
bioreactor (Applikon, 2L working volume) with cells from a
15   logarithmic pre-culture grown in shake flask. The pre-culture
and bioreactor experiments were performed in ExCell VPRO (JRH
Biosciences). The split ratio for inoculation into the
bioreactor was at least 1:6, and the seeding cell
concentration was about 0.3×10^6 cells/ml.
20

Results
Fig. 7 shows the results of the modified feed on clone 1 in
bioreactor versus a batch control. The maximum viable cell
number reached $10\text{-}12 \times 10^6$ ml^{-1} and viable cell numbers were
25   maintained between 8 and 10×10^6 cells ml^{-1} until the end of the
culture at day 19 (Fig. 7A). Antibody yields increased 3-fold
from 0.4 g L^{-1} in the batch to 1.3 g L^{-1} in the fed-batch
process (Fig. 7B).
Osmolality and ammonia reached 430 mOsm Kg^{-1} and 16 mmoles L^{-1}
30   respectively in these feed cultures, levels that have been
reported as having negative effects on culture performance and
product quality. It may therefore be that the decrease in

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viable cell numbers observed towards the end of the process
was due at least in part to these factors.

Fig. 8 shows the results of the feed strategy on clone 2 in 2L
bioreactors. Maximum viable cell numbers reached 10-11 x 10^6

5   ml^{-1} and 7-9 x 10^6 ml^{-1} were maintained until the end of the
culture at 19 days. Antibody yields were increased 3-fold from
0.5 g L^{-1} to 1.5 g L^{-1}.

A third clone expressing another, again unrelated, antibody
was subjected to the same batch process and the fed-batch

10   process with the same feed strategy. Fig. 10 shows the results
of the feed strategy on this·clone 3 in shake flask. Maximum
viable cell numbers reached 14 x 10^6 ml^{-1} and 10-12 x 10^6 ml^{-1}
were maintained until the end of the culture on day 17.
Antibody yields were increased 3-fold from 0.7 g L^{-1} to 2.1 g

15   L^{-1}.

The feed strategy therefore improves the yield for different
clones that each express a different antibody, indicating that
the process according to the invention is generically

20   applicable.

It is therefore an aspect of the invention to provide a
process comprising the feed strategy according to the
invention, wherein the yield of a produced protein is
increased at least 1.5 x, preferably at least 2 x, more

25   preferably at least 2.5 x, still more preferably at least 3x
over the yield in the batch process.

The specific productivity (q_{Ab}) of the cells used in the
present invention was approximately between 12-18 pg

30   antibody/cell/day. In some instances the q_{Ab} was around 10 pg
antibody/cell/day, and in other instances values up to about
25 pg antibody/cell/day were observed with the cells and

methods of the present invention. In the batch cultures this
decreased significantly before maximum cell numbers were
reached, coinciding with depletion of nutrients, which was
approximately after 7 days, whereas in fed-batch cultures this
5 specific productivity was kept at this level until 2-3 days
after the last feed addition, which amounts to around 16-18
days, according to a process of the invention.

Product Quality

10 In the experiments described above, product quality was
checked by various methods, including iso-electric focusing,
SDS-PAGE, MALDI-TOF mass spectrometry and HPAEC-PAD. In all
cases the produced antibody basically showed a human-type
glycosylation and the structural integrity of the produced
15 antibodies was very good, irrespective of the process used,
and very similar to that reported in (Jones et al, 2003),
where both cell numbers and product yields were lower.
Therefore, the increased yields obtainable by processes of the
invention were not obtained at the cost of a significant
20 decrease in product quality.
Protein A purified IgG produced from batch and fed-batch
cultures was analysed by MALDI-MS. Material produced by PER.C6
cells from batch cultures showed a galactosylation profile
similar to that shown by IgG purified from human serum and no
25 hybrid or high mannose structures were identified in either
batch or fed-batch produced material. The average percentage
of glycans terminating in 0, 1 and 2 galactose residues
(G0:G1:G2) from all the batch cultures tested was 29, 54 and
17 % respectively. This can be compared to CHO and hybridoma
30 produced antibody, which is often predominantly in the G0
form. For example, Hills et al (1999) reported a

galactosylation profile (G0:G1:G2) for an antibody produced in
NS0 and CHO cells.

Antibody produced in the fed-batch process showed a reduced
level of galactosylation compared to the batch (Fig. 9). The
5   percentage of G0 glycoforms increased from 29 to 49%, while
the G1 and G2 glycoforms decreased from 54% and 17% to 42% and
9% respectively. This decrease in galactosylation was probably
due to the high (up to 16 mM) ammonia concentrations at the
end of the fed-batch cultures. However, the level of
10   galactosylation in the antibody produced by the fed-batch
process in PER.C6 cells was still higher than typically seen
in batch-produced antibodies from CHO for example (Hills et al
1999). Isoelectric focusing (IEF) and SDS-PAGE revealed no
significant differences between the material produced by batch
15   or fed-batch cultures (data not shown) and in all cases,
aggregation was below 3%.

Despite relatively low $Y_{amm/gln}$ values, the high viable cell
concentrations resulted in a supply of glutamine in the feed
such that the ammonia accumulated up to 16 mmoles L^{-1}. Whilst
20   this did not result in a drop in the viable cell
concentration, batch cultures initiated in the presence of
NH_4Cl showed that concentrations above 9 mmole L^{-1} negatively
affected growth rates and maximum cell concentrations.
Furthermore, glycosylation was also somewhat affected (see
25   Fig. 9). It may therefore be beneficial to reduce ammonia
accumulation, e.g. according to a method described below.

Two areas for attention in the process described so far are
the high levels of ammonia and osmolality. A large contributor
30   to the increase in osmolality came from the VPRO (medium)
concentrates. An approach to reduce this osmolality is
therefore to identify which of the medium component groups

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(vitamins, trace elements, inorganic salts, growth factors etc) are important to culture performance and remove those that are not important. This should benefit the process not only by reducing the osmolality of the feed but also by

5   removing any potentially deleterious components and by allowing the optimization of addition of the most important components. It would also reduce the cost of the feed. Reduction in ammonia accumulation may be achieved by more strictly controlling glutamine addition. This can be done

10 based on the calculations of the specific consumption and cell numbers as described supra. This can be achieved by continuously pumping in glutamine at an appropriate rate, matched to the viable cell concentration and the cell specific rate of utilization, so that residual glutamine concentrations

15 in the medium are maintained at a constant low level, such as between 0.2 and 1.5 mM, preferably between 0.5 and 1.0 mM. Another approach that may be possible for the cells according to the invention is the removal of glutamine from the feed when the ammonia concentration reaches a certain point -e.g.

20 in one or more of the feeds subsequent to the first feed- so that the cells are forced to switch to glutamine synthesis using ammonia and glutamate and the glutamine synthetase pathway. This approach is not generally possible for cell types such as BHK and CHO as glutamine depletion often results

25 in rapid and widespread cell death and transfer to glutamine-free conditions often requires a period of adaptation. However, in batch cultures of the cells according to the present invention, the viable cell concentration continued to increase for two days after the depletion of glutamine and

30 culture viability was not significantly affected, suggesting that there may be sufficient flux through the glutamine synthetase pathway at least to maintain the culture.

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Spent medium analysis of the most optimized fed-batch culture
(examples in Fig. 7, 8) showed that only cystine was depleted
during the process. A further modification of the amino acid
feed according to the invention is therefore an increase in
5   the cystine concentration, e.g. to 0.3-0.35 mmoles/l or even
up to 0.6 mmoles/l for every 10 x 10^6 cells/ml.

Example 5: improved (fed-)batch process.
        Feed concentrates developed for fed-batch processes may
10   also be used to supplement culture media for use in an
improved batch process. Supplementing a culture medium with at
least one of the feed additions from a fed-batch process has
been shown by others to improve batch yields. A similar
approach of supplementing culture media with feed concentrates
15   may also be used to reduce the number of feed additions during
a fed-batch process, thereby simplifying the process, as also
shown by others.
        The present invention discloses feed strategies for cells
that have been immortalized by adenovirus E1 sequences, such
20   as PER.C6 cells. It is shown herein which components become
limiting in a fed-batch process, and the amounts of as well as
the ratio between the components that can be added to improve
yields in a fed-batch process are disclosed herein. This
information is used in this example to provide an improved
25   batch process. It is assumed that such a culture will contain
about 10 x 10^6 cells/ml, as this is around the number of cells
that has been observed in the batch and fed-batch cultures of
the invention. In the fed-batch experiments, 6 feeds were
added, with concentrations of the components as in Tables 1 or
30   2. The addition of 10%-60% of the total (i.e. the total of all
6 feeds together) feed, preferably 20%-40% of the total feed,
results in an improved batch process, because the nutrients

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will become depleted later during the culture, and hence the
yields will go up because of prolonged productivity compared
to the straight batch process disclosed above, where no
additions are made to the culture medium. The components can

5   be added directly to the culture medium at any stage prior to
depletion of nutrients from the medium, but are preferably
added prior to start of the culture so that no other additions
have to be made during the process (improved batch process),
which makes the process very simple. Of course, this may be

10   combined with extra additions of certain components later
during the process (fed-batch process), in which case less
additions have to be made to make the process than in the fed-
batch process disclosed above, thereby providing a simpler
fed-batch process. It is therefore another embodiment of the

15   invention to provide a method for producing a product in cells
immortalized by adenovirus E1 sequences, wherein said cells
are cultured in a culture medium, characterized in that the
following components are added to the culture medium in the
following amounts: glucose (3.6 – 21.6 mmoles/l, preferably

20   7.2 – 14.4 mmoles/l), glutamine (6.8 – 40.9 mmoles/l,
preferably 13.6 – 27.2 mmoles/l), leucine (0.40 – 2.4
mmoles/l, preferably 0.79 – 1.6 mmoles/l), serine (2.31 – 13.9
mmoles/l, preferably 4.62 – 9.24 mmoles/l), isoleucine (0.3 –
1.8 mmoles/l, preferably 0.6 – 1.2 mmoles/l), arginine (0.28 –

25   1.66 mmoles/l, preferably 0.55 – 1.10 mmoles/l), methionine
(0.14 – 0.83 mmoles/l, preferably 0.28 – 0.55 mmoles/l),
cystine (0.15 – 0.9 mmoles/l, preferably 0.3 – 0.6 mmoles/l),
valine (0.27 – 1.62 mmoles/l, preferably 0.54 – 1.08
mmoles/l), lysine (0.26 – 1.58 mmoles/l, preferably 0.53– 1.06

30   mmoles/l), threonine (0.18 – 1.08 mmoles/l, preferably 0.36 –
0.72 mmoles/l), asparagine (0.06 – 0.36 mmoles/l, preferably
0.12 – 0.24 mmoles/l), tyrosine (0.078 – 0.47 mmoles/l,

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preferably 0.16 - 0.31 mmoles/l), histidine (0.06 - 0.36
mmoles/l, preferably 0.12 - 0.24 mmoles/l), phenylalanine
(0.012 - 0.072 mmoles/l, preferably 0.024 - 0.048 mmoles/l),
tryptophan (0.036 - 0.22 mmoles/l, preferably 0.072 - 0.14
5   mmoles/l) and phosphate (0.45 - 2.7 mmoles/l, preferably 0.9 -
1.8 mmoles/l). The amounts between brackets are 10%-60%,
preferably 20%-40%, of the amounts of 6x the feeds of Table 2.
Preferably, also culture medium concentrate (10x, 50x, or
other suitable concentrates can be used) is added to an end
10 concentration of between 0.15x - 0.9x, preferably between 0.3x
- 0.6x. Preferably the culture medium in these embodiments is
ExCell VPRO medium. An amount of 0.5, 1, 1.5, 2, 2.5, 3, 3.5
or 4 single feeds (a single feed being an amount as disclosed
in Table 1 or 2) is added to culture medium, and simple batch
15 processes for culturing the cells at around 10 x 10^6 cells/ml
and producing product (e.g. antibody) according to the
invention are performed with the thus fortified media, to
determine the optimum amount of component additions. Improved
batch processes giving the highest product yields are expected
20 when about 20%-40% of the total feed of a fed-batch process
according to the invention are provided to the culture medium
prior to culturing, i.e. somewhere between 1 to 2.5 single
feeds. Of course, more fine-tuning of the amount is possible
once a beneficial range of added components is established by
25 these experiments. Of course, when the cell numbers are
different, the component addition can again be adapted. For
instance, if the cells are cultured at a density of only 5 x
10^6 cells/ml, addition of an amount of only half the amount
above would be required, as is clear to the person skilled in
30 the art.

Table 1

Components	Final Concentration (after addition) (per 10 x 10^6 cells/ml) (mmoles L^{-1})
Glucose	6.00
Glutamine	1.75
Leucine	0.60
Serine	0.55
Isoleucine	0.45
Arginine	0.42
Methionine	0.23
Cystine	0.14
Valine	0.45
Lysine	0.40
Threonine	0.33
Glycine	0.33
Asparagine	0.15
Tyrosine	0.14
Histidine	0.11
Penylalanine	0.10
Tryptophan	0.02
Phosphate	0.70
Calcium	0.02*
LongR3 IGF-1	50 ug/L*
Long EGF	50 ug/L*
Insulin	20 ug/L*

*optionally present

Table 2

Components	Final Concentration (after addition) (per 10 x 10^6 cells/ml) (mmoles L^{-1})	
	First Feed	Subsequent Feeds
Glucose	6.00	6.00
Glutamine	2.60	1.75
Leucine	0.66	0.66
Serine	1.10	0.55
Isoleucine	0.50	0.50
Arginine	0.46	0.46
Methionine	0.23	0.23
Cystine	0.25	0.23
Valine	0.45	0.45
Lysine	0.44	0.44
Threonine	0.30	0.30
Asparagine	0.10	0.10
Tyrosine	0.13	0.13
Histidine	0.10	0.10
Penylalanine	0.02	0.02
Tryptophan	0.06	0.06
Phosphate	0.75	0.75
10X VPRO Concentrate	0.25X	0.25X

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40

CLAIMS

1. A method for the culturing of cells immortalized by
   adenovirus E1 sequences, said cells capable of growing in
5 suspension, comprising the steps of:
   a) determining at least once during the culturing of the
   cells the concentration of at least one medium component
   selected from the group consisting of glucose, glutamine
   phosphate, leucine, serine, isoleucine, arginine,
10 methionine, cystine, valine, lysine, threonine and
   glycine,
   b) adding components to the medium during the culturing
   of the cells at or prior to the depletion of at least one
   of the components of which the concentration was
15 determined in step a), wherein
   the components added at least comprise glucose,
   glutamine, phosphate, leucine, serine, isoleucine,
   arginine, methionine and cystine.

20 2. A method according to claim 1, wherein the at least one
   medium component of which the concentration is determined
   in step a) are chosen from the group consisting of
   glucose, glutamine, phosphate, leucine, serine,
   isoleucine, arginine, methionine and cystine.

25

3. A method according to claim 1 or 2, wherein in step a)
   the concentration is determined of at least two of said
   medium components.

30 4. A method according to any one of claims 1-3, wherein the
   components added in step b) further comprise one or more
   of valine, lysine, threonine, glycine, asparagine,

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tyrosine, histidine, phenylalanine, tryptophan,
phosphate, calcium, LongR3 IGF-1, Long EGF and insulin.

5. A method according to any one of claims 1-4, wherein the
components in step b) are added in an end concentration
in mmoles/l of freshly added component per 10×10^6
cells/ml of between 4.0 and 8.0 for glucose, 0.44 and
0.88 for leucine, 0.37 and 1.47 for serine, 0.33 and 0.67
for isoleucine, 0.31 and 0.61 for arginine, 0.15 and 0.31
for methionine, and 0.1 and 0.6 for cystine.

6. A method according to claim 5, wherein in step b)
glutamine is further added to an end concentration of
1.17 and 3.47 mmoles/l of freshly added glutamine per 10
$\times 10^6$ cells/ml.

7. A method according to claim 6, wherein the components in
step b) are added more than one time, further
characterized in that as a result of the first addition
the end concentration of freshly added glutamine is
higher than as a result of a subsequent addition.

8. A method according to claim 5, wherein glutamine is
further added essentially continuously such that the
residual concentration of glutamine in the medium is
maintained between 0.2 and 1.5 mM, preferably between 0.5
and 1.0 mM.

9. A method according to any one of claims 5-8, wherein in
step b) the following components are further added to an
end concentration in mmoles/l of freshly added component
per 10×10^6 cells/ml of between 0.3 and 0.6 for valine,

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0.29 and 0.59 for lysine, 0.2 and 0.4 for threonine.

10. A method according to claim 9, wherein in step b) the
    following components are further added to an end
5   concentration in mmoles/l of freshly added component per
    10×10^6 cells/ml of between 0.067 and 0.13 for
    asparagine, 0.087 and 0.17 for tyrosine, 0.067 and 0.13
    for histidine, 0.013 and 0.027 for phenylalanine, and
    0.04 and 0.08 for tryptophan.
10

11. A method according to any one of claims 1-10, wherein
    the addition of the components in step b) is performed at
    between 48 hours and just prior to depletion of at least
    one of the medium components the concentration of which
15 was determined in the previous step.

12. A method according to any one of claims 1-11, wherein
    said steps are repeated at least once.

20 13. A method according to any one of claims 1-12, wherein
    said cells are derived from retina cells.

14. A method according to claim 13, wherein said cells are
    derived from human embryonic retina cells as represented
25 by the cells deposited under ECACC no. 96022940.

15. A method according to any one of claims 1-14, wherein
    the cells are grown to a cell density of at least 9×10^6
    per ml.
30
16. A method according to any one of claims 1-15, wherein
    the cells produce a product that is harvested.

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17. A method according to claim 16, wherein said product is
    a recombinant protein.

5   18. A method according to claim 17, wherein said recombinant
    protein is an immunoglobulin that is secreted into the
    culture medium to a level of at least 500 mg per liter.

19. A method according to claim 18, wherein said recombinant
10   protein is an immunoglobulin that is secreted into the
    culture medium to a level of at least 1000 mg per liter.

20. A method according to anyone of the preceding claims,
    wherein the cells are in suspension during culturing.
15

21. A culture of cells immortalized by adenovirus E1
    sequences, characterized in that said culture comprises
    at least 10×10^6 cells/ml.

20 22. A culture of cells according to claim 21, characterized
    in that said culture comprises at least 12×10^6
    cells/ml.

23. A culture of cells according to claim 22, characterized
25   in that said culture comprises at least 15×10^6
    cells/ml.

24. A culture of cells according to claim 23, characterized
    in that said culture comprises at least 20×10^6
30   cells/ml.

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25. A culture of cells according to claim 24, characterized
    in that said culture comprises at least 30 x 10^6
    cells/ml.

26. A culture of cells according to claim 25, characterized
    in that said culture comprises at least 40 x 10^6
    cells/ml.

27. A culture of cells according to any one of claims 21-26,
    wherein at least 80% of the cells are viable.

28. A culture of cells according to any one of claims 21-27,
    wherein culture medium is exchanged at a rate of about
    0.2-3 culture volumes/day.

29. A culture of cells according to any one of claims 21-28,
    wherein cells in said culture express a recombinant
    protein.

30. A culture of cells according to claim 29, wherein said
    cells produce at least 10 pg protein/cell/day.

31. A culture of cells according to claim 21, wherein said
    culture is a batch culture and wherein cells in said
    culture express a recombinant immunoglobulin with a yield
    of at least 500 mg/l.

32. A culture of cells according to claim 30, wherein said
    yield is at least 700 mg/l.

33. A culture of cells according to claim 29 or claim 30,
    wherein said culture is a perfusion or fed-perfusion

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culture and wherein said recombinant protein is an
immunoglobulin that is expressed with a yield of at least
150 mg/l/day.

5   34. A culture of cells according to any one of claims 21-33,
       wherein said cells are derived from human embryonic
       retina cells as represented by the cells deposited under
       ECACC no. 96022940.

10   35. A culture of cells according to any one of claims 21-34,
       wherein said culture is a suspension culture.

       36. A method for producing a product in cells immortalized
       by adenovirus E1 sequences, said cells being in a culture
15   medium, wherein said product is chosen from the group
       consisting of a recombinant protein, a virus, and a
       recombinant adenovirus with a deletion in the E1 region,
       characterized in that
       at least Glutamine, Glucose, Phosphate, Leucine, Serine,
20   Isoleucine, Arginine, Methionine and Cystine are added to
       the culture medium.

       37. A method according to claim 36, further characterized in
       that said cells reach a cell concentration of at least 20
25   x 10^6, preferably at least 30 x 10^6 viable cells/ml at
       least part of the time in said process.

       38. A method for producing a product in cells immortalized
       by adenovirus E1 sequences, wherein said cells are
30   cultured in a culture medium, characterized in that the
       following components are added to the culture medium per
       liter: 3,6-21.6 mmoles glucose, 6.8-40.9 mmoles

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glutamine, 0.40-2.4 mmoles leucine, 2.31-13.9 mmoles
serine, 0.3-1.8 mmoles isoleucine, 0.28-1.66 mmoles
arginine, 0.14-0.83 mmoles methionine, 0.15-0.9 mmoles
cystine, 0.27-1.62 mmoles valine, 0.26-1.58 mmoles

5   lysine, 0.18-1.08 mmoles threonine, 0.06-0.36 mmoles
asparagine, 0.078-0.47 mmoles tyrosine, 0.06-0.36 mmoles
histidine, 0.012-0.072 mmoles phenylalanine, 0.036-0.22
mmoles tryptophan and 0.45-2.7 mmoles phosphate.

10   39. A method according to claim 38, wherein the amounts of
the components added to the culture medium per liter are:
7.2-14.4 mmoles glucose, 13.6-27.2 mmoles glutamine,
0.79-1.6 mmoles leucine, 4.62-9.24 mmoles serine, 0.6-1.2
mmoles isoleucine, 0.55-1.10 mmoles arginine, 0.28-0.55

15   mmoles methionine, 0.3-0.6 mmoles cystine, 0.54-1.08
mmoles valine, 0.53-1.06 mmoles lysine, 0.36-0.72 mmoles
threonine, 0.12-0.24 mmoles asparagine, 0.16-0.31 mmoles
tyrosine, 0.12-0.24 mmoles histidine, 0.024-0.048 mmoles
phenylalanine, 0.072-0.14 mmoles tryptophan and 0.9-1.8

20   mmoles phosphate.

40. A method according to claim 38 or claim 39, wherein
further culture medium concentrate is added to an end
concentration of between 0.15x - 0.9x, preferably between

25   0.3x - 0.6x.

41. A method according to any one of claims 38-40, wherein
said components are added to the culture medium prior to
culturing the cells.

30

1 / 1 2

Fig 1

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Fig. 2

Fig.3A

4 / 1 2

Fig.3B

Fig.3C

Fig.3D

Fig.3E

Fig. 4

A.

B.
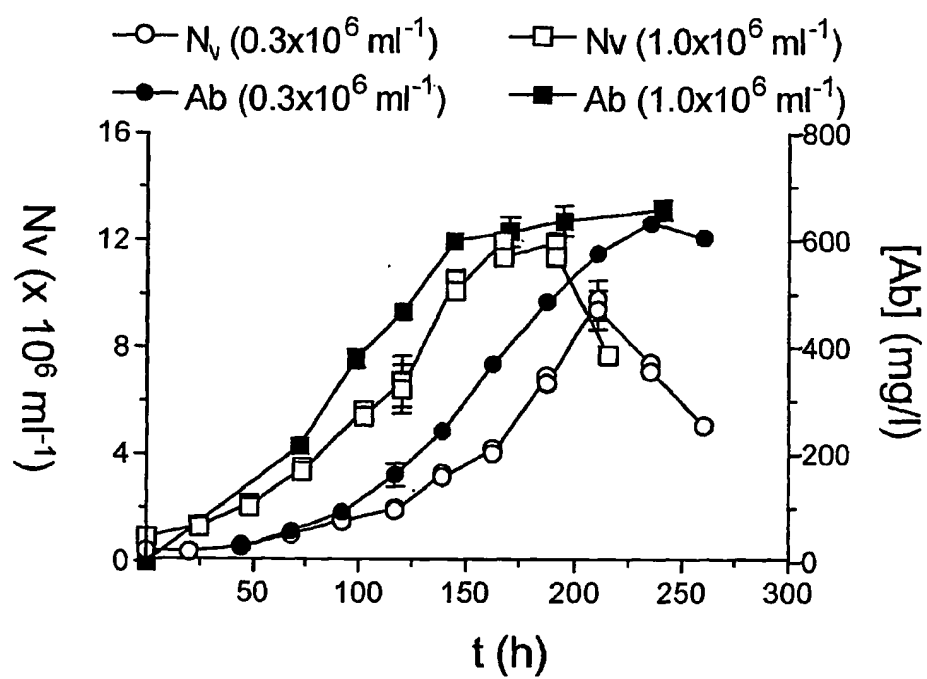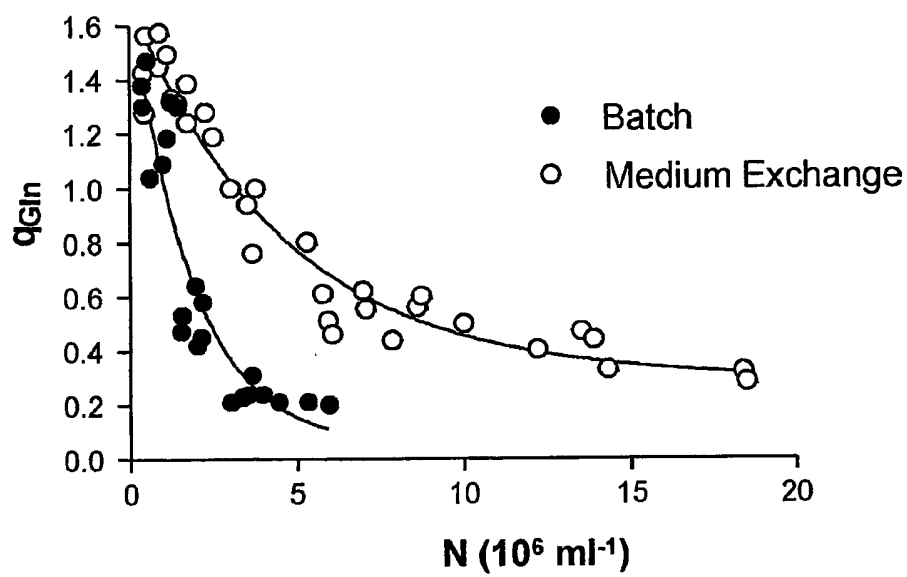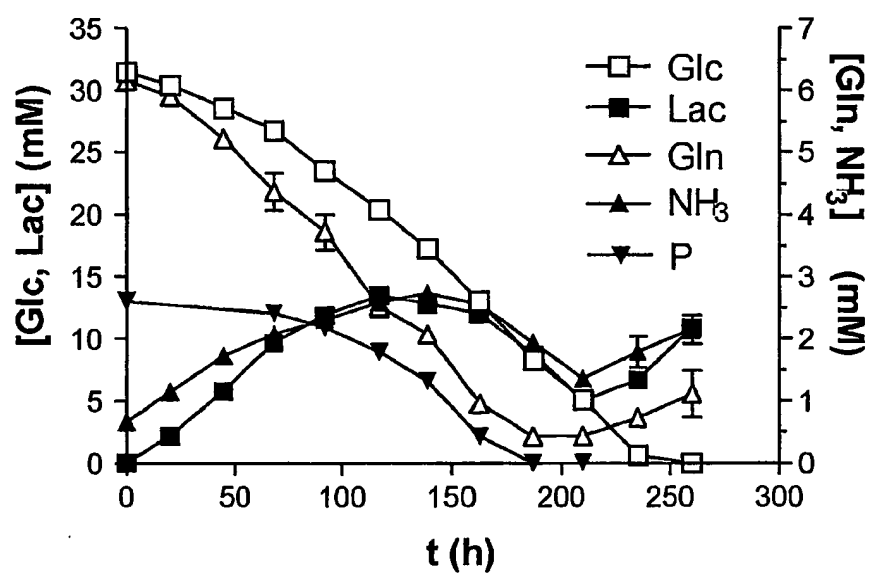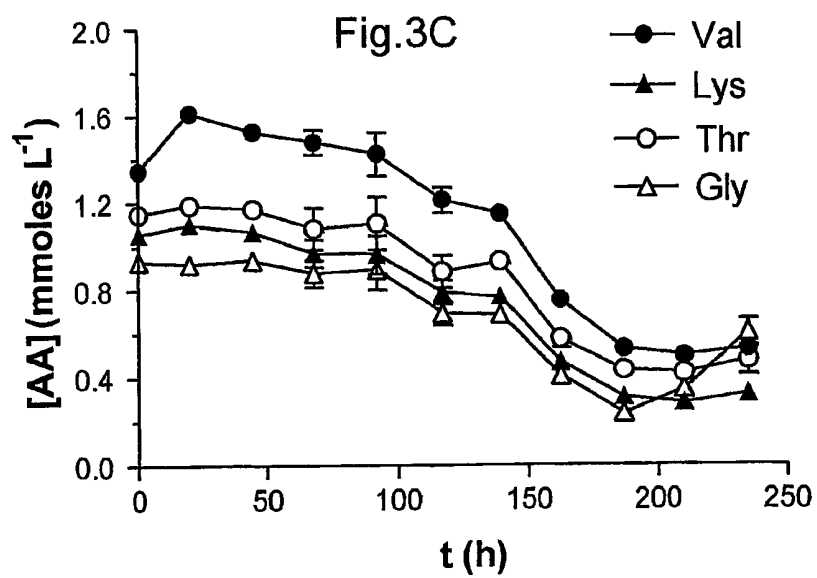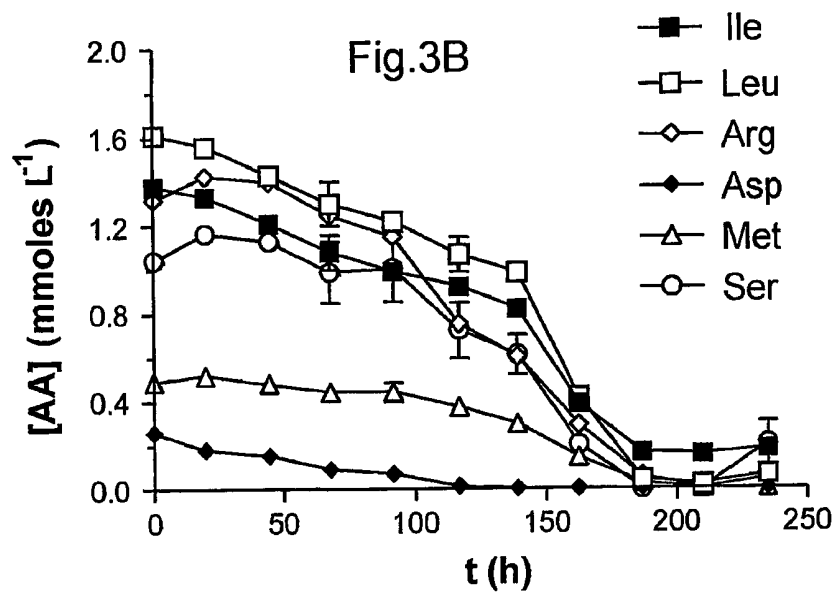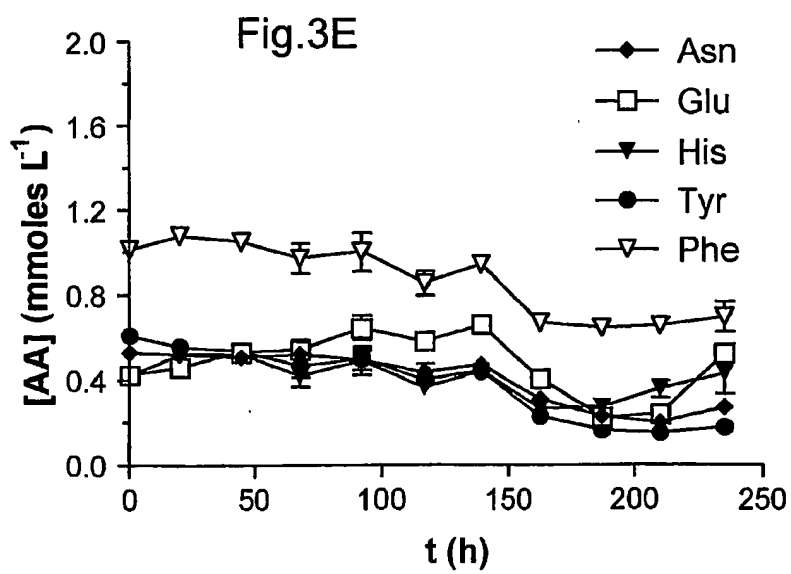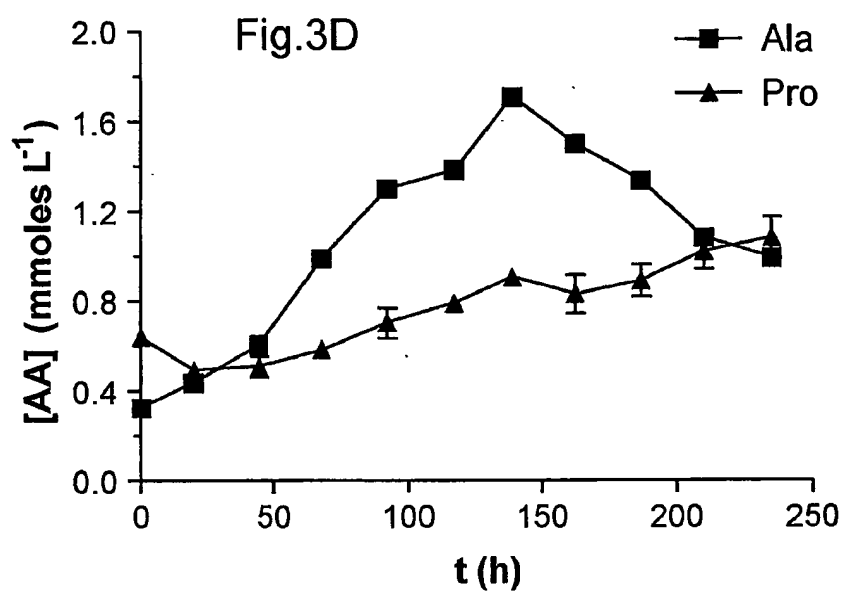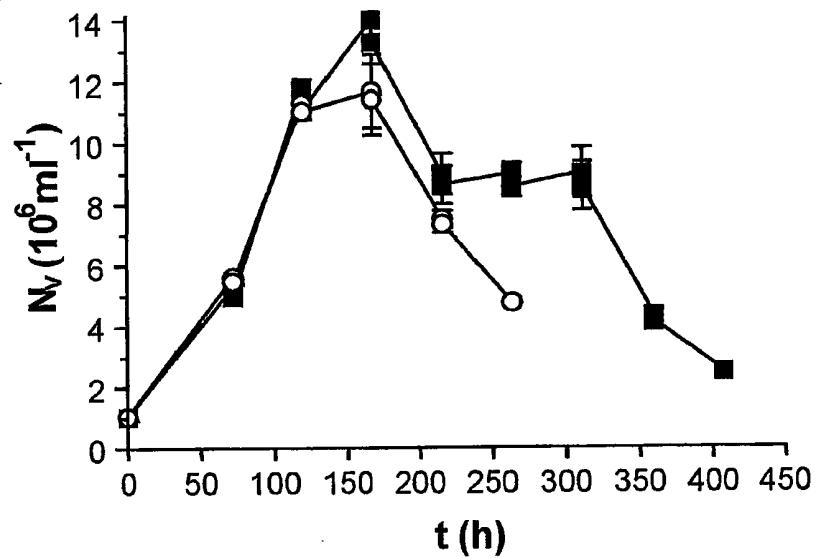
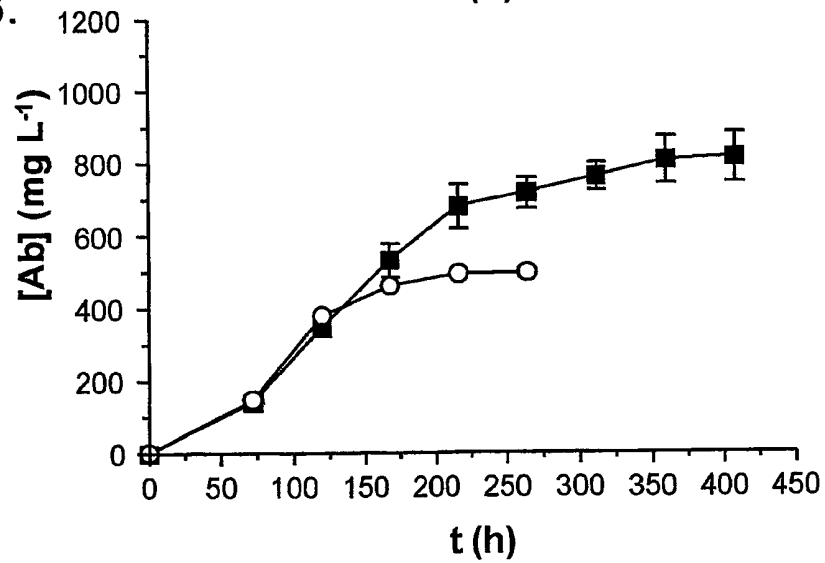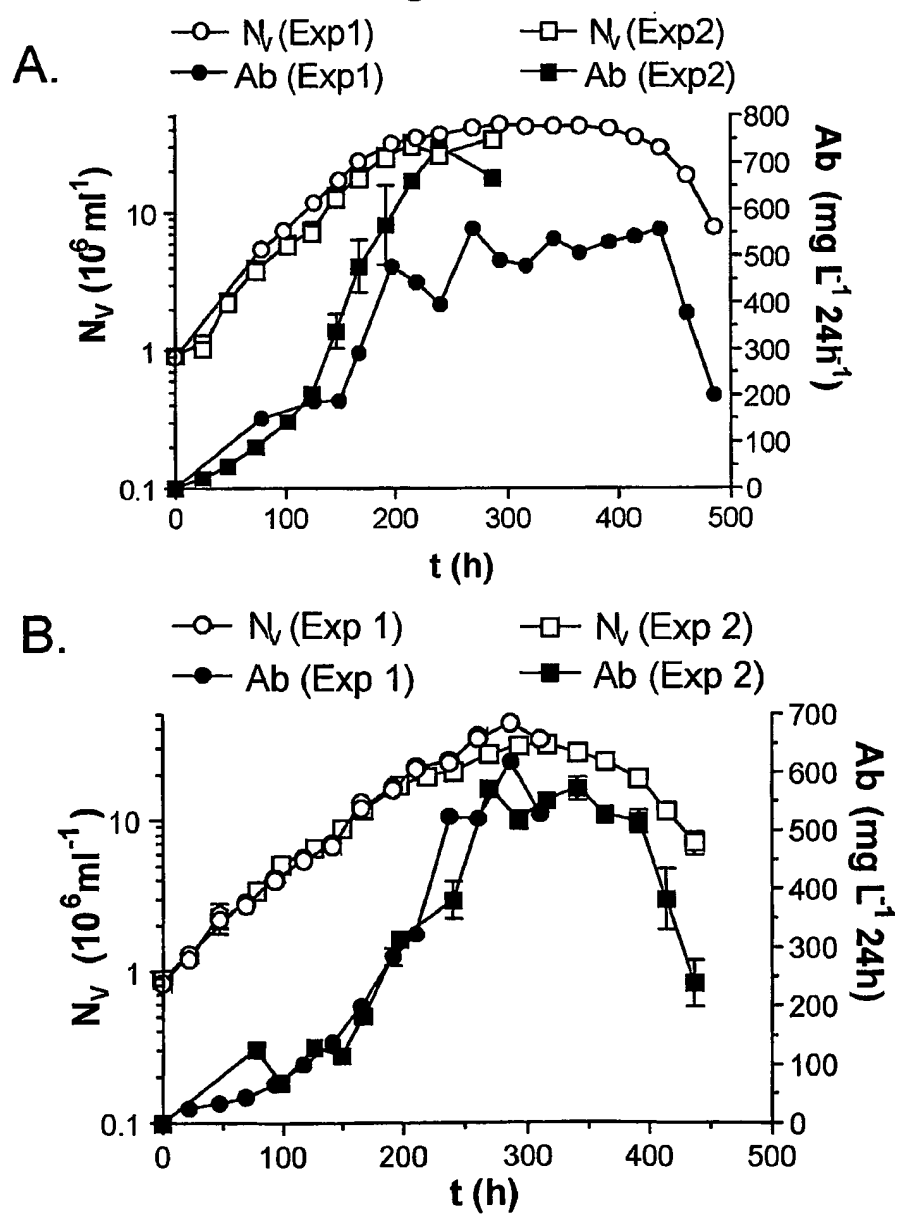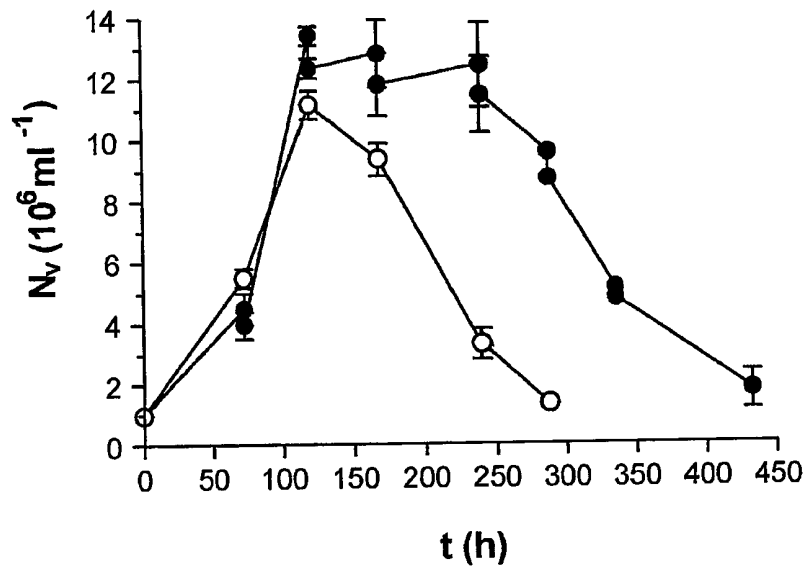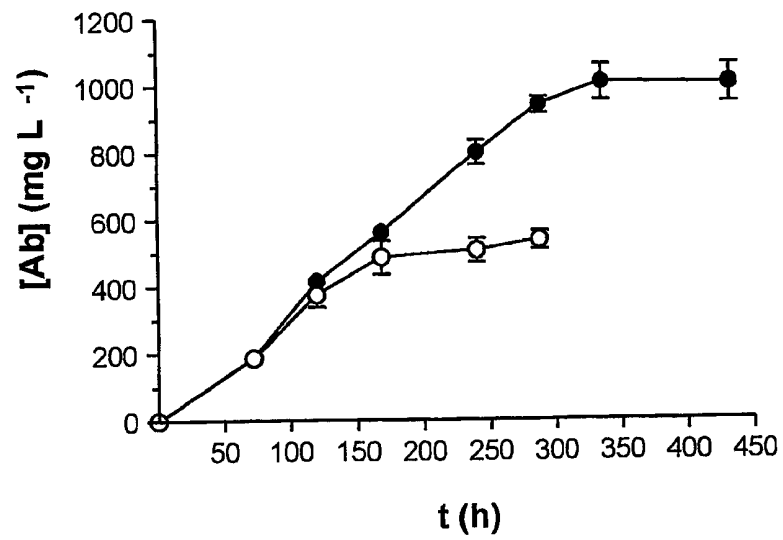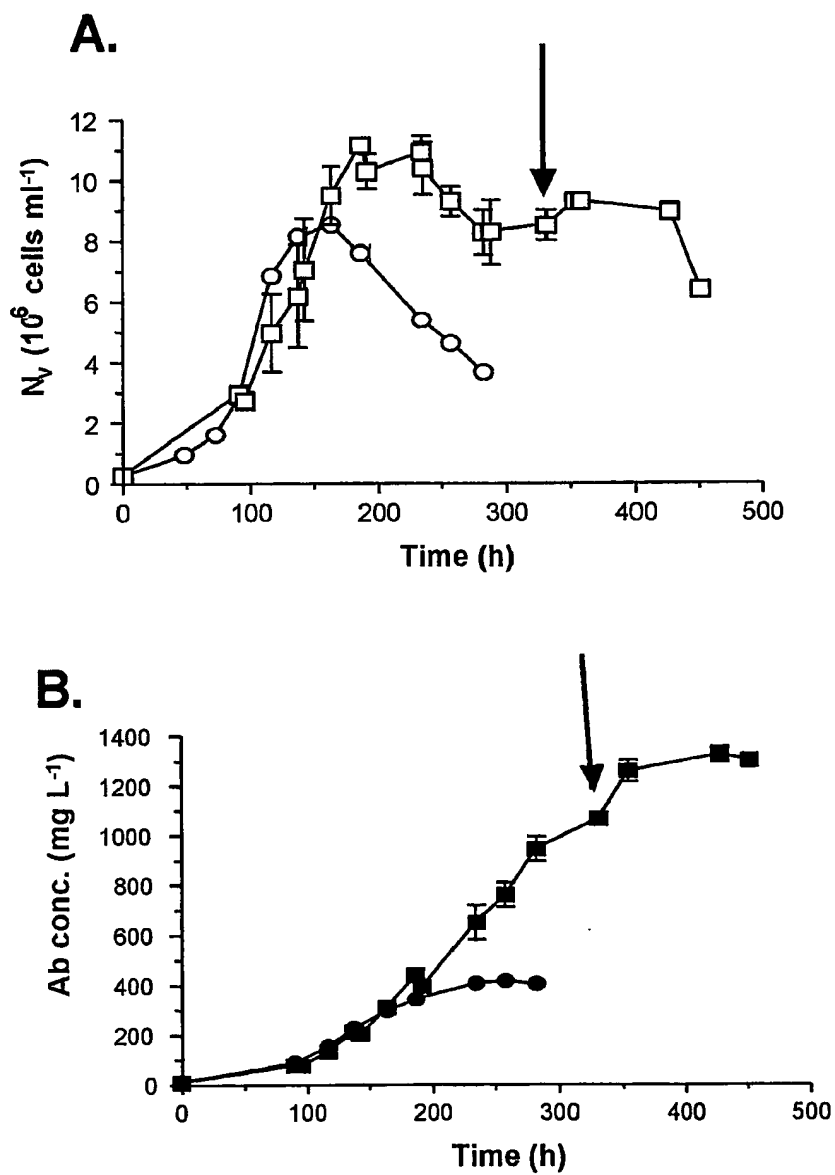## Fig. 5

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Fig. 6

A.

B.

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Fig. 7

A.

B.

Fig. 8
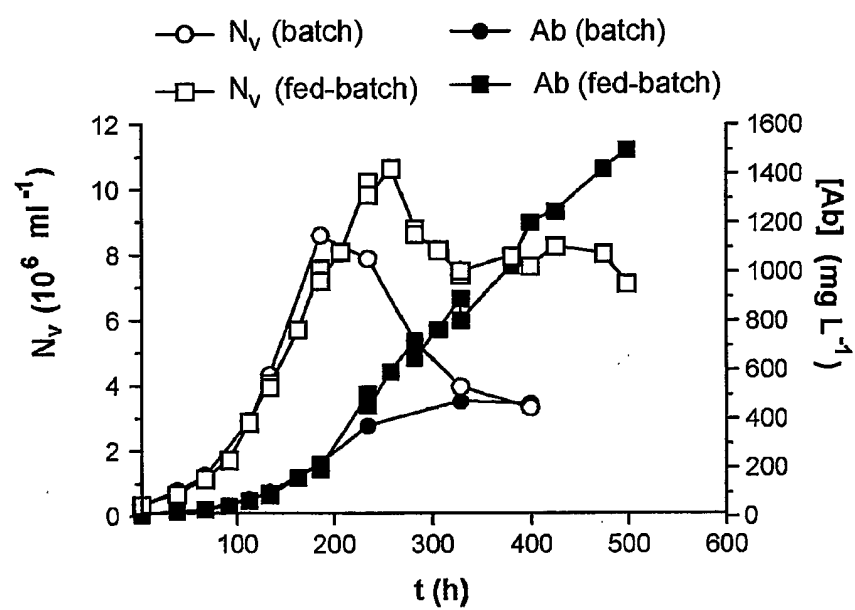
1 1 / 1 2

Fig. 9
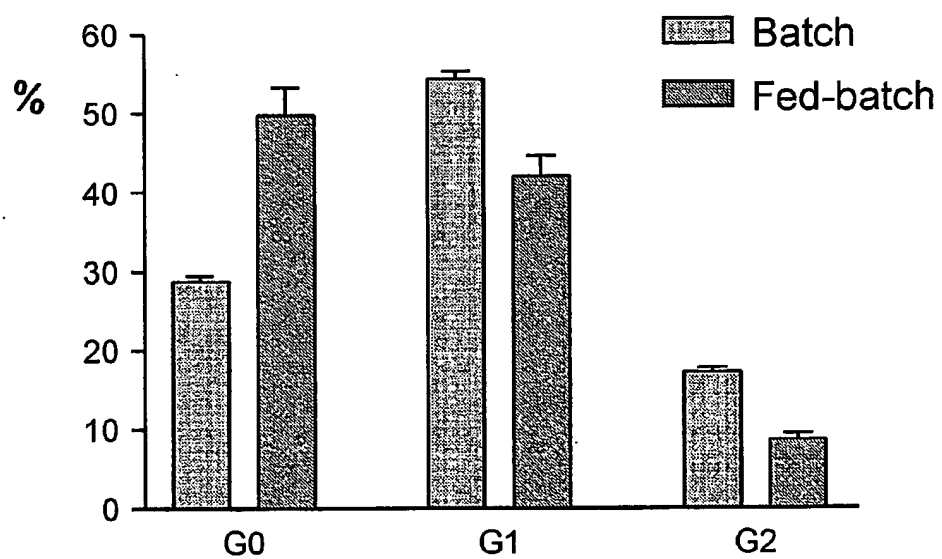
1 2 / 1 2

Fig. 10
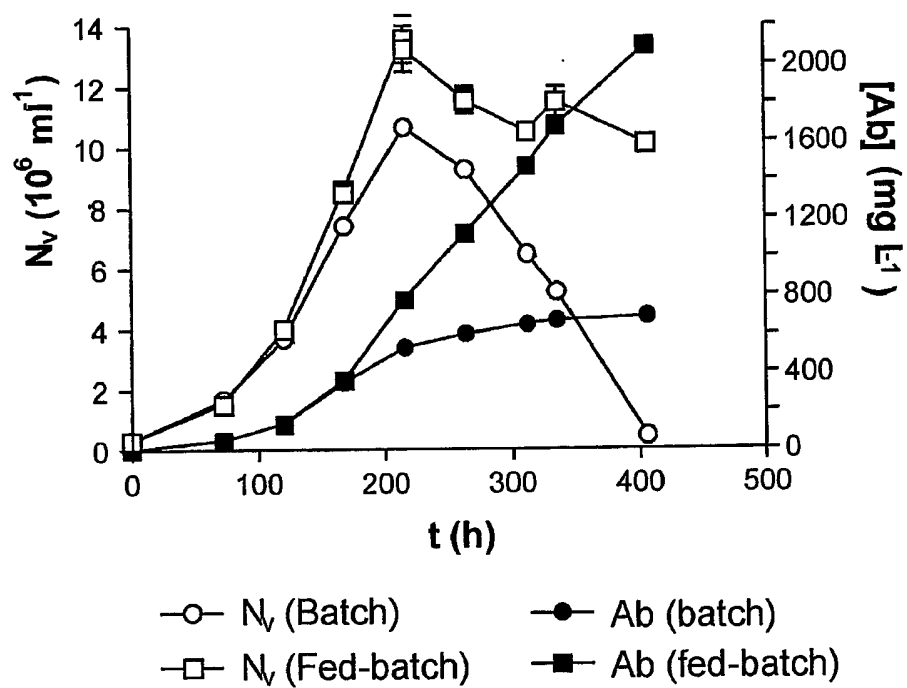
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, MEDLINE, EMBASE, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BOUT B: "PER.C6TM AS PRODUCTION PLATFORM FOR HUMAN MONOCLONAL ANTIBODIES" HUMAN ANTIBODIES, AMSTERDAM, NL, vol. 12, no. 1-2, 8 October 2003 (2003-10-08), page 30, XP009023644 ISSN: 1093-2607 the whole document	21-41
X	JONES D ET AL: "High level expression of recombinant IgG in the human cell line PER.C6" BIOTECHNOLOGY PROGRESS, XX, XX, vol. 19, 14 January 2003 (2003-01-14), pages 163-168, XP002256988 ISSN: 8756-7938 figure 5	21-41

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| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 September 2004	01/10/2004

| Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016 | Authorized officer Friedrich, C |

Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JONES D H ET AL: "PER.C6 CELL LINE FOR HUMAN ANTIBODY PRODUCTION CRUCELL'S TECHNOLOGY MAINTAINS HUMAN GLYCOSYLATION PATTERNS" GENETIC ENGINEERING NEWS, MARY ANN LIEBERT, NEW YORK, US, vol. 22, no. 10, 15 May 2002 (2002-05-15), page 50,54, XP009018783 ISSN: 1270-6377 the whole document	1-41
A	XIE L ET AL: "SERUM-FREE SUSPENSION CULTIVATION OF PER.C6 CELLS AND RECOMBINANT ADENOVIRUS PRODUCTION UNDER DIFFERENT PH CONDITIONS" BIOTECHNOLOGY AND BIOENGINEERING. INCLUDING: SYMPOSIUM BIOTECHNOLOGY IN ENERGY PRODUCTION AND CONSERVATION, JOHN WILEY & SONS. NEW YORK, US, vol. 80, no. 5, 5 December 2002 (2002-12-05), pages 569-579, XP001155285 ISSN: 0006-3592 figures 2,4	1-41
A	EP 1 108 787 A (CRUCELL HOLLAND B V) 20 June 2001 (2001-06-20) paragraph '0068!	1-41
T	XIE LIANGZHI ET AL: "Large-scale propagation of a replication-defective adenovirus vector in stirred-tank bioreactor PER.C6TM cell culture under sparging conditions." BIOTECHNOLOGY AND BIOENGINEERING, vol. 83, no. 1, 5 July 2003 (2003-07-05), pages 45-52, XP002296365 ISSN: 0006-3592 page 46	1-41
T	PHAM PHUONG LAN ET AL: "Large-scale transient transfection of serum-free suspension-growing HEK293 EBNA1 cells: Peptone additives improve cell growth and transfection efficiency." BIOTECHNOLOGY AND BIOENGINEERING, vol. 84, no. 3, 5 November 2003 (2003-11-05), pages 332-342, XP002296366 ISSN: 0006-3592 figure 9	1-41

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such
   an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
   searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

                               ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2004)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

This International Searching Authority found multiple (groups of)
inventions in this international application, as follows:

1. claims: 1-20

   Strategy for feeding E1 adenovirus immortalized cells
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2. claims: 21-35

   Culture of E1 adenovirus immortalized cells comprising at
   least 10 e7 cells.
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3. claims: 36-41

   Culture method for E1 adenovirus immortalized cells
   comprising a certain culture medium

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